Isolation, identification and characterization of calcifying bacteria and its role on biomineralization of calcium carbonate and its biological applications

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Abstract
Biomineralization is a significant process carried out by living organisms in which minerals are produced through the hardening of biological tissues. Herein, the current study focus on calcium carbonate precipitation, as part of biomineralization, to be used in applications for CO_2 sequestration, material technology, and other fields. A strain B. licheniformis, isolated from marine water, was investigated for its ability to produce urease and induce calcium carbonate precipitation in a metabolic process. It was discovered that *B. licheniformis*, resisted high concentrations of urea up to 60 g/L. In order to optimize the calcification process of *B. licheniformis*, the Calcium carbonate precipitation media is used respectively, pH of 10, and culture time of 96 h. Using X-ray diffraction and Scanning Electron Microscopy analysis, the calcium carbonate polymorphs produced by *B. licheniformis*, were proven to be mainly calcite. The results of this research provide evidence that *B. licheniformis*, can biologically induce calcification and suggest that *B. licheniformis*, may play a potential role in the synthesis of new biominerals and in bioremediation or biorecovery.

Keywords: biomineralization, *B. licheniformis*, calcium carbonate

Introduction
Emission control of Green House gas, carbon dioxide (CO_2), is a big challenge to the scientists and technologists all over the world. A large amount of CO_2 is emitted by different anthropogenic sources causing pollution to the environment. This increased level of CO_2 has not only adverse effects on the atmosphere but also the agricultural land and surface water bodies. This new technique, is generally applied where buildings need protection from creaks and it stabilizes slopes near sand dunes or river edges against liquefaction in a consequence of weathering. Biogrouted solutions are now applied to creaks remediation, inhibition of dental caries and to stop damage of the marble rocks on Tajmahal's (one of the world's seventh wonder) roof, by spreading ureolytic bacteria on the marble tiles.

Because of the amounts of CO2 in the atmosphere, scientists are now seeking new ways to eliminate carbon dioxide or to prevent it from increasing. One alternative is to create new equilibrium conditions in carbon and calcium cycles by sequestering and storing CO_2 in the form of stable carbonates. As a result, research is now being carried out on the use of microorganisms (e.g., bacteria) to facilitate carbonate precipitation in certain environments. It has been found that such microorganisms contribute to CO_2 fixation by precipitating it in the form of insoluble carbonates that are extremely stable in most natural habitats.

In the present study, a urease synthesis bacterial strain *B. Licheniformis* was isolated from active sludge and characterized by 16S rRNA gene sequence analysis, and the calcium carbonate polymorphs produced by the strain *B. Licheniformis* were analyzed using X-ray diffraction. In addition, the biological calcification induced by the strain *B. Licheniformis* under different conditions was determined. The results suggest that *B. Licheniformis* has potential application in bioremediation of contaminated environments and material technology.

Materials and Methods
Bacteria Isolation and Culture Conditions
Calcium carbonate precipitating strains were isolated from rameshwaram marine sediment. The sample was suspended in a filter sterilized saline solution (0.85% NaCl), diluted appropriately and plated on calcium carbonate precipitation media (CCP) containing (per liter) 20 g of urea, 2.12 g $NaHCO_3$,10g NH_4Cl,3g of Nutrient broth, 30 mM $CaCl_2$, 20 g agar, pH 8.5. The plates were then incubated at 28 °C for 7 days, and the appearing colonies were assessed under a stereo-microscope. The positive individual colonies were finally selected based on their visual crystal formation and purified by repeated streaking on the calcium carbonate precipitation media with $CaCl_2$ removed.

DNA Extract, PCR Amplification and Sequencing
Bacterial genomic DNA was extracted from pure culture with the fast spin kit (Invitrogen) following the manufacturer's instructions. Amplification of 16S rRNA gene was performed in 50 µL of reaction mixture containing 0.25 mM each primer of 27 f (5'-GTTTGATCCTGGCTCAG-3') and 1492r (5' TACCTTGTTACGACTT-3'), 0.2 mM dNTP, 1.5 mM $MgCl_2$,5µL of Taq buffer, and 5 U Taq DNA polymerase (Invitrogen, USA), 10-20 ng template DNA. PCR was then performed on a thermalcycler under the following conditions: 95 °C for 5 min, 35 cycles of 50 s at 95 °C, 50 s

at 45 °C and 1.5 min at 72 °C, followed by a final extension for 10 min at 72°C. The PCR products were visualized on an agarose gel, and the bands with the corrected size were excised and purified using the Wizard SV gel purification protocol (Promega, USA). The partial 16S rRNA fragment was sequenced on an ABI 3730 automated DNA sequencer (Applied Bio-systems).

Phylogenetic Analysis

Phylogenetic affiliation of each 16S rRNA sequence was initially queried by BLAST search to suggest the closest relatives against the GenBank database. The sequences were then aligned with their relatives using Clustal W, and phylogenetic trees were constructed from a matrix of pair-wise genetic distances by the maximum-parsimony algorithm of the MEG 4 software. Three partial sequences of 16S rRNA genes from the strain, *B. Licheniformis* isolated from marine sediment, have been deposited in the GenBank database under accession numbers: KF378645, KF378646, KF378647, respectively.

Urease Activity Assay

All the isolates were tested for their urease activity on the urea agar media containing 1.0 g of pancreatic digest gelatin, 1.0 g of dextrose, 5.0 g of sodium chloride, 2.0 g of monosodium phosphate, 20.0 g of urea, 12.0 mg of phenol red, 15.0 g of agar, and the final pH was adjusted to 6.8. 0.5 µL cell suspension of each candidate strain (10 6 cells/mL) was inoculated on the urea agar media, and the plates were incubated at 28 °C for 1-2 days. The urease activity was resolved on the media to the extent of the indication of the pink-red color, which specifically represents the generation of alkaline conditions that are attributed to the production of ammonia via urease activity on urea. An Escherichia coli strain was chosen as the negative control.

Test for Calcium Carbonate Solubilization

Strains isolated from the calcium carbonate precipitation agar plates were tested for their solubilization capability of calcium carbonate on the media (CCS) containing (per liter) 0.5 g of yeast extract, 10 g of dextrose, 5g of $CaCl_2$, 0.5g of $(NH4)_2 SO_4$, 5g of $Ca_3 (PO_4)_2$, 0.2 g of KCl, s0.1 g of $MgSO_4$, 0.0001 g of $MnSO_4$and 0.0001 g of $FeSO_4$, 20 g agar, pH 7.0, and grown at 28 °C for 5 days. The solubilization capability of calcium carbonate was quantified by measuring the diameter of the clear halo around a colony.

Calcium Carbonate Precipitation and Collection

For calcium carbonate precipitation and collection, bacteria were grown aerobically in 100 mL of liquid calcium carbonate precipitation media in 500 mL Erlenmeyer flasks and incubated at 28°C for 60 h. The control consisted of uninoculated liquid calcium carbonate precipitation medium. At each time point and after the incubation, the whole culture was centrifuged at 10,000 g for 1 min. The pellet, which included calcium carbonate precipitate and the bacteria cells, was resuspended in 50 mL TE buffer (10 mM Tris, 1 mM EDTA pH 8.5). Lysozyme was added at a final concentration of 1 mg/mL and the cell suspension was incubated at 37 °C for 1 h to digest the bacteria cell wall. The cell debris was removed by centrifugation and the pellet was washed with sterile distilled water (pH 8.5), then air dried at 37 °C for 24 h. The pellet was weighed to estimate the amounts of carbonate crystals precipitated by the different strains and subjected to the following analyses.

X-ray Diffraction Analysis (XRD)

X-ray diffraction (XRD) was used to determine the mineralogy of calcium carbonate precipitation induced by different bacteria. The collected dry precipitation of calcium carbonate was crushed using a mortar and pestle, then homogenized with ethanol. The powdered sample was back-packed into an aluminum sample holder and analyzed using XRD on a Panalytical X'Pert PRO MPD (Cu-K|Á) at the Nuclear Industry Geological Analysis and Testing Research Center (Beijing, China). Instrument parameters were set to 40-kV accelerating voltage and 35-mA current. Scans were run from 20° to 60° 2Θ at a scanning speed of 0.01 °/s. The peak in the d (112) was used to determine the calcite minerals.

Scanning Electron Microscopy (SEM)

Morphology of calcium carbonate precipitation was observed by scanning electron microscopy (SEM Hitachi S-450). The collected carbonate crystals were mounted directly into the SEM stubs and sputter-coated with a gold/palladium mixture (Hitachi HUS-5GB coating unit). Scanning was performed under the condition of accelerating voltage at 25 kV. Cell number, pH and chemical analytic methods In order to determine the correlation of calcium carbonate formation to the parametric changes during the growth phase of *B. Licheniformis*, parameters such as cell number, pH and ammonia were monitored at constant time intervals. At each time point of post incubation, a 0.5 mL aliquot of the culture was taken from the flask, appropriately diluted, then spread on the nutrition broth agar (per liter,5gofenzymatic digest gelatin,3gofbeef extract, 15 g of agar) and incubated at 28 °C for 24 h to determine the cell numbers. Calcium carbonate precipitation was deter-mined as described above and the supernatant was used to determine the pH and the concentration of ammonia. pH was measured using a pH indicator (PB-10, Sartorius AG). Ammonia released in the medium as a result of urea hydrolysis was determined by the spectrophotometric method.

Results and Discussion
Isolation of Bacteria Involved in Inducing Calcium Carbonate Precipitation

Bacterial strain was isolated from calcium carbonate precipitation agar plates, all of which could induce the precipitation of calcium carbonate under those conditions. Microscopy revealed that precipitation started with a scattered white spot circling the bacteria colony, then developed into a hard gray-white crystal covering the colony with an encircling scattered white spot appearing after 7 days (fig. 1).

Fig 1: Calcium Carbonate precipitating bacterial strain isolated from sea water

Phylogenetic Analysis of the Isolated Candidate Strain

The isolate were identified and characterized by sequencing of 16S rDNA. These sequences were BLAST searched against the Gen Bank database using the BLASTN program (Fig. 2).
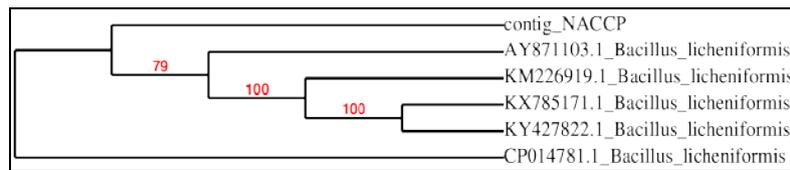

Fig 2: Neighbor-joining tree based on partial 16S rRNA gene sequences showing the phylogenetic relationship of the 20 isolate and their closest relatives. The phylogenetic tree was generated using approximately 1,400 bp of 16S rRNA sequence by the neighbor-joining method. Reference strains used in the tree can be retrieved with their accession numbers in GenBank. Scale bar equals approximately 2% nucleotide divergence.

Characterization of the Phylogenetic Distinct Strain

Characterization of phylogenetic distinct strain *B. Licheniformis* were investigated and characterized by their growth rate, capability of inducing calcium carbonate precipitation, urease activity, and calcium carbonate solubilization ability. Microbial induced calcium carbonate precipitation by urea hydrolysis was investigated extensively. The bacterium converts urea into ammonia by producing the enzyme urease, thus increasing the environmental pH and subsequently inducing calcium carbonate precipitation. The isolated strains possessed the urease activity when tested in the urea agar media *B. Licheniformis* generated higher urease activity, whereas the E. coli strain did not show any purple color surrounding the inoculated site, which indicates a lack of urease activity. This urease activity assay result, together with the result, implied that the mass of calcium carbonate precipitation was directly linked to the urease activity, with higher urease activity causing more calcium carbonate precipitation. Therefore, strain *B. Licheniformis* was chosen for further analyses. However, no calcium carbonate formed when plates were inoculated with E. coli control. To determine whether the isolates play roles in the deterioration of limestone by solubilizing calcium carbonate, carbonate solubilization capability was tested on the calcium carbonate solubilization media. *B. Licheniformis* dissolved calcium carbonates and formed a clear, circular halo around the inoculation site. These data suggested that the mechanism of calcium carbonate precipitation induced by bacteria may change with variations in environmental conditions.

SEM and XRD Analyses of Microbically-Induced Calcium Carbonate Precipitation

Precipitations collected on the 7th day were analyzed by XRD. The results showed that the isolated strain, induced the formation of calcite, which was the only crystal assayed in the XRD spectra (Fig. 3). Morphologies of crystals induced by B. *Licheniformis* were observed under SEM microscopy, and the results showed the morphologies of crystals induced by B. *Licheniformis* is observed (Fig. 4). Basically, five different morphologies of crystals, the cubic crystal, the rhombic crystal, the polygonal plate-like crystal, the spherical crystal, and the irregular shaped crystal, were observed. The cubic, the rhombic and the polygonal plate-like crystal were the three main shapes of crystals induced by the isolates, while the spherical and the irregular shaped crystals were less common. Different morphologies of crystal showed the different properties of aggregated minerals described as follows. The surfaces of cubic shaped crystals were smooth. The rhombic-shaped and the polygonal plate like crystals generally presented well defined faces and edges with accumulation of plate like structures. The spherical crystals were formed by accumulation of granular composition with a rough surface Microbial induced calcite precipitation.
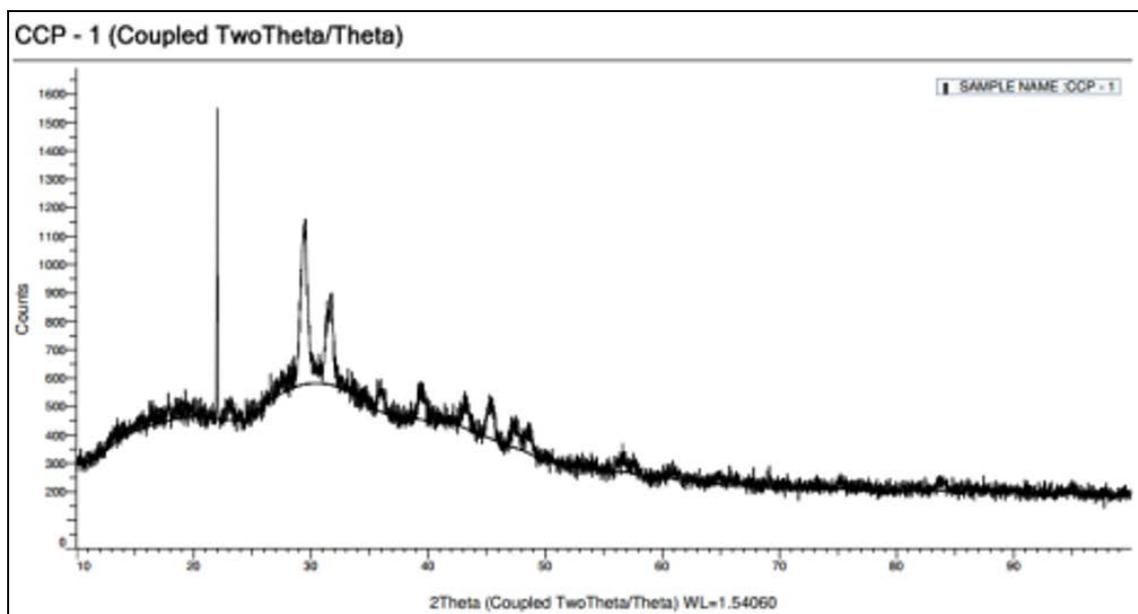

Fig 3: XRD spectra of the calcium carbonate crystals induced by B. Licheniformis, calcite. From top to bottom
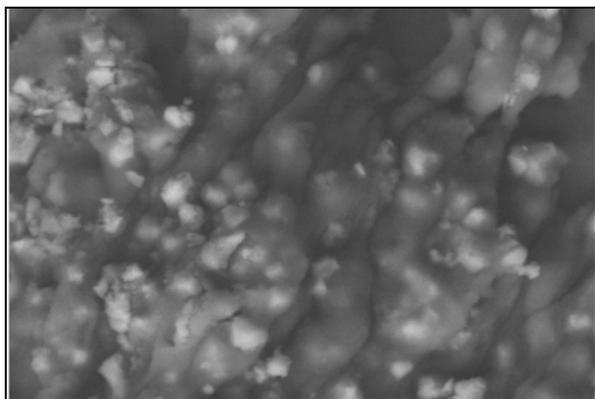
Fig 4: SEM micrographs revealing the morphology Y of calcite crystals induced by B. Licheniformis

Based on our observation and analyses, isolated strain induces calcite precipitation in the liquid media. To determine the correlation of calcium carbonate formation with the metabolic parameter changes on the growth phase of B. *licheniformis*, several parameters, including pH, cell number, ammonium ion concentration and mass of calcium carbonate, were monitored. The amount of calcium carbonate precipitation appeared to maintain a positive correlation with the growth of B. *licheniformis*. The pH quickly increased from the initial pH of 8.3 to 9.4 in the first 12 h of inoculation. While in the stage of log phase growth, B. *licheniformis* maintained robust growth, and the concentration of ammonium ions, which is believed to have contributed to the rise of pH, increased to 608 mM. Relatively more calcium carbonate precipitation was precipitated during this period. When the growth of B. *licheniformis* was in the stationary phase after 12 h of incubation, the pH gradually increased to 9.6 from 9.4 and the ammonium ion concentration slightly increased to 746 mM. During this period, the rate of calcium carbonate precipitation was lower than that of the first phase. The calcium carbonate precipitation tended to reach plateaus, with a production of 96 mg/L in this phase. However, in the control experiment without bacteria, the pH of the media increased only slightly from 8.3 to 8.4, and the concentration of ammonium ions remained relatively stable. With such slight alkalinity of the media, only trace calcium carbonate precipitation was collected in the control experiments.

Conclusions

The present study demonstrates that B. *licheniformis*, a Gram-Positive isolate of active sludge, induces urea hydrolyzation and calcium carbonate precipitation. The strain B. *Licheniformis* resisted concentrations of urea up to 60 g/L and simultaneously produced urease, and when B. *Licheniformis* was cultured with both urea and Ca^{2+}, calcium carbonate precipitation occurred. Optimized calcite precipitation culture conditions were determined to be: concentrations of 50μM Ni^{2+} and 60 g/L urea, pH value of 10, and culture time of 96 h. We further discovered that calcite was the dominant calcium carbonate form of the calcium carbonate polymorphs produced by B. *Licheniformis*. The results of this research suggest that B. *licheniformis* is a potential candidate for synthesis of new biominerals and has potential application in environmental bioremediation and biorecovery.

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